IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of:

Frank, *et al.*

Appl. No.: 09/873,409

Filed: June 5, 2001

For: **A Gene Encoding a Multidrug Resistance Human P-Glycoprotein Homologue on Chromosome 7p15-21 and Uses Thereof**

Art Unit: 1642

Examiner: Yu M.

Atty. Dkt.: 7570/73298

#20
KD
10/16/03

Declaration Under 37 C.F.R. §1.132

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Sir:

The undersigned, Markus Frank, MD, declares as follows:

1. I am, at present, Instructor in Medicine at Harvard Medical School and an Associate Physician in the Department of Medicine at Brigham & Women's Hospital in Boston, Massachusetts. In addition, I am named as an inventor on the above-captioned application.

2. I have an MD from the University of Heidelberg, Germany, and have conducted research in the area of P-glycoprotein biology for the past 5 years. A Curriculum Vitae establishing my credentials as an expert in this field is attached.

3. All of the research described herein was conducted in my laboratory under my direction. I am therefore thoroughly familiar with the experiments that were performed and the results that were obtained.

4. I have reviewed the above-noted application and the claims pending therein. I understand that the Examiner has rejected claims based upon the allegation that there is insufficient evidence that the proteins encoded by the claimed polynucleotides have a credible utility. In particular, the Examiner argues that assertions that the proteins act as transporters conferring drug resistance on cells is not credible. For the reasons set forth below, I respectfully disagree.

5. P-glycoproteins (P-gp) and related members of the ATP-binding cassette (ABC) superfamily of active transporters mediate multidrug resistance in mammalian cancers(1-8) and serve physiologic transport(9-17), differentiation(17-19) and survival(20,21) functions in non-malignant cell types. Several members of the ABC superfamily of transporters, including ABCB1 (MDR1) P-glycoprotein(22), the ABCG2 (Bcrp1) transporter(23), and the ABCB5 P-glycoprotein transporter cloned and characterized in my laboratory(17) (i.e. the protein of SEQ ID NO-2 encoded by the nucleotide of SEQ ID NO-10 of the above-captioned application) are expressed at high levels on stem and progenitor cell populations(17,22,23) and are characterized by their capacity to transport the fluorescent dyes rhodamine 123(17,24-26) and Hoechst 33342(27-30) out of cells. The studies described below, which include peer-reviewed results published in the

scientific literature(17), were conducted using ABCB5 P-glycoprotein, one of the gene products of the human chromosome 7p15-21 P-glycoprotein gene, and identified by the protein of SEQ ID NO-2 encoded by the nucleotide of SEQ ID NO-10 of the above-captioned application.

Based upon the studies described below, it is my conclusion that it is more likely than not that the ABCB5 P-glycoprotein gene encodes a protein which, like the multidrug resistance protein ABCB1 (MDR1), serves as a transporter contributing to drug resistance. It is also my opinion that one of ordinary skill in molecular biology and biochemistry would draw the same conclusion. My conclusions are based upon: a) homology studies demonstrating a high degree of structural homology between ABCB5 and ABCB1 (MDR1) P-glycoprotein; b) cell expression studies demonstrating that ABCB5 P-glycoprotein expression is limited to specific cell types that include primary human melanocytes, drug resistant human melanoma cancer cells, clinical human malignant melanoma primary tumors and tumor metastases; c) expression studies showing that ABCB5 P-glycoprotein, like ABCB1 (MDR1), is expressed in progenitor cells; d) cell localization studies showing that ABCB5 P-glycoprotein is localized on cellular plasma membranes (consistent with a transport function) and is increased in drug resistant cells; e) studies directly demonstrating that ABCB5 P-glycoprotein is an active cellular drug efflux transporter; and f) studies demonstrating that ABCB5 P-glycoprotein blockade or depletion of chemoresistant ABCB5-expressing tumor cells sensitizes tumor cells to chemotherapy. The results of these studies are summarized in the sections below and in the attached figures referred to therein. Figure Legends are provided in attached Appendix I. The methodology used is described more fully in Appendix II and references are provided in Appendix III.

I. Homology Studies

We used the National Center for Biotechnology Information (NCBI) tblastn application to compare conserved amino acid sequences derived from the known ABCB1 (MDR1) P-glycoprotein structure against the NCBI non-redundant homo sapiens nucleotide sequence database dynamically translated in all reading frames and identified a novel human P-glycoprotein-encoding gene at the chromosome 7p15-21 locus by sequence homology. Using a gene-specific oligonucleotide primer pair and PCR amplification of reverse transcribed total mRNA isolated from primary human epidermal melanocytes (HEM), or the G3361 human malignant melanoma cell line and its cisplatin (CDDP)-resistant variant G3361/CDDP(31), we amplified a cDNA encoding a novel human P-glycoprotein family member, designated ABCB5 (Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 5) P-glycoprotein(17). Comparison of the encoded 812 amino acid ABCB5 primary sequence revealed that the molecule is highly homologous (73 %) to both of the known human P-glycoprotein isoforms ABCB1 (MDR1) and ABCB4 (MDR3) (54% and 56% amino acid identity, respectively; Fig. 1), its closest human relatives. Primary structure analysis using the PIR International Protein Family Classification system algorithm confirmed the encoded protein to be a novel MDR P-glycoprotein member of the ABC transport protein superfamily(17).

II. Expression studies

A. Expression in Specific Cells Including Cancer Cells and Multidrug Resistant Cancer Cells

Using a gene-specific oligonucleotide primer pair and PCR amplification of reverse transcribed total mRNA isolated from primary HEM, or the G3361 human malignant melanoma cell line and its cisplatin (CDDP)-resistant variant G3361/CDDP(31), we amplified a 2906 base cDNA (Fig. 2) encoding a novel human P-glycoprotein family member, designated ABCB5 P-glycoprotein. ABCB5 P-gp mRNA transcripts were not detected in peripheral blood mononuclear cells (PBMC) or additional non-melanoma human tumors, including the MCF-7 breast cancer and SCC-25 squamous cell carcinoma cell lines, indicating a selective tissue expression pattern of ABCB5 P-glycoprotein among human physiologic tissues and malignant cancers. Sequencing of the 2906 base cDNA demonstrated that the cDNA contained the complete coding sequence for ABCB5 P-glycoprotein, since the 467-base 5'-untranslated region contained five in-frame stop codons upstream of the translation initiation codon(17). Furthermore, using a ABCB5 gene-specific oligonucleotide primer pair as above and PCR amplification of reverse transcribed total mRNA isolated from a series of clinical human melanoma tumor biopsies, including primary tumors and a tumor metastasis, we demonstrated that ABCB5 P-glycoprotein is also expressed in such clinical human cancers, in addition to chemoresistant tumor cultures.

B. Location on Cell Membrane

Western analysis, using a mouse anti-ABCB5 monoclonal antibody (mAb) (clone 3C2-2D12) generated in the laboratory against a synthetic immunogen derived from an extracellular loop-associated ABCB5 epitope, detected a 89 kDa protein band of the predicted size (ExPASy server

Compute pI/Mw Tool) in HEM, G3361 melanoma cells and ABCB5 gene-transfected MCF-7 breast cancer cells, but not lacZ gene-transfected MCF-7 controls (Fig. 3A), demonstrating specific reactivity of this antibody with ABCB5 P-glycoprotein. The lipofectamine-mediated gene transfection efficiency varied from 5 to 20% in MCF-7 cells in repeat experiments (Fig. 3B). To characterize cellular localization and membrane topology, we performed indirect surface immunostaining and flow cytometry of non-permeabilized ABCB5 gene-transfected MCF7 cells or lacZ gene-transfected MCF-7 controls. Labeling with anti-ABCB5 P-glycoprotein 3C2-2D12 mAb, raised against an epitope contained in amino acid residues 493-508 of the molecule, revealed 14% immunopositivity versus $ABCB5^-$ controls (Fig. 3C, M1 gate), consistent with the determined transfection efficiency. These findings demonstrate a surface plasma membrane localization of ABCB5 P-glycoprotein and support a predicted (TMHMM1.O software algorithm) membrane topology of ABCB5 P-glycoprotein characterized by five transmembrane helices flanked by both extra- and intracellular ATP-binding domains (Fig. 3D)(17).

C. Augmentation in Drug Resistant Cells

We next examined ABCB5 P-glycoprotein surface expression in native HEM, the G3361 melanoma cell line and its cisplatin-resistant variant G3361/CDDP, which transcribe ABCB5 mRNA as determined by PCR analysis. Indirect immunostaining using 3C2-2D12 mAb(17) revealed ABCB5 P-glycoprotein to be expressed on the plasma membrane of 11%

of HEM, 3% of G3361 melanoma cells and, significantly augmented, on 13% of CDDP-resistant G3361/CDDP melanoma cells (Fig. 4A, M1 gate).

D. Expression on Progenitor Cells

We next examined whether ABCB5 P-glycoprotein marked a progenitor phenotype-expressing subpopulation. Among purified $CD133^+$ cells(32,33), which constitute a stem cell subset in human skin(34) and comprised from 0.2 to 0.5 % of cells among HEM cultures in our studies as determined by flow cytometry ($p<0.05$), ABCB5 P-glycoprotein high-expressing cells were markedly enriched, with 56% of cells expressing the molecule versus 16% of cells among unseparated controls (Fig. 4B). $CD133^+ABCB5^+$ cells did not express the hematopoietic progenitor cell antigen CD34(35), as determined by flow cytometry(17). Immunofluorescence examination of purified, cultured $CD133^+$ HEM revealed a focal expression pattern of ABCB5 P-glycoprotein, with immunostaining localizing predominantly to the cellular poles and additional focal membrane domains (Fig. 4C, 1-5). Unexpectedly, and in contrast to unseparated HEM cultures, $CD133^+ABCB5^+$ HEM also comprised abundant numbers of multinucleated cells, including cells with four, three and two nuclei. These findings demonstrated that ABCB5 P-glycoprotein marks a distinct cell subset among cultured HEM characterized by mono- and multinucleated cells of $CD133^+$ progenitor phenotype(17).

III. Transport Function

When assessing the role of ABCB5 P-glycoprotein in fluorescent rhodamine-123 transport, a hallmark function of the closely related ABCB1 (MDR1) P-glycoprotein, ABCB5 gene transfection induced a de novo rhodamine-123 efflux capacity in 15% of MCF-7 breast carcinoma compared to lacZ gene-transfected MCF-7 controls (Fig. 5A, M1-gated rhodamine^{low} phenotype), consistent with the transfection efficiency and demonstrating that plasma membrane-expressed ABCB5 P-glycoprotein functions as a rhodamine efflux transporter(17). In addition, we examined the role of ABCB5 P-glycoprotein in the cellular transport of the fluorescent chemotherapeutic drug doxorubicin in ABCB5-expressing human G3361 melanoma cells. Our results showed that ABCB5 P-glycoprotein blockade by anti-ABCB5 mAb treatment significantly enhanced cellular retention of doxorubicin in 11 % of cells compared to control mAb-treated cultures (Fig. 5B, M1 gate), directly demonstrating a role of ABCB5 P-glycoprotein in the cellular transport of this chemotherapeutic agent.

IV. Chemoresistance

Based on our finding that ABCB5 P-glycoprotein expression was specifically augmented in cisplatin-resistant G3361/CDDP melanoma cells, we examined whether ABCB5 P-glycoprotein contributed to cisplatin resistance in these cells. In the absence of ABCB5 P-glycoprotein inhibition, a 50 μM cisplatin concentration was needed to achieve 50% cell killing (LC50) of G3361 cells, compared to 90 μM in the resistant G3361/CDDP cell line (Fig. 6A). Anti-ABCB5 P-glycoprotein Ab treatment sensitized G3361/CDDP cells to cisplatin treatment, causing a

45% reduction in the LC50 to levels comparable to those observed in less resistant G3361 cells (Fig. 6B). The observed resistance reversal effect was strongest at the highest cisplatin concentration tested (200 μM) (69% of control survivors killed, $p<0.05$ using the unpaired student t test), suggesting a preferential effect on the ABCB5 P-glycoprotein high-expressing tumor cell subset. Ninety percent cell killing (LC90), not achievable in untreated G3361 or G3361/CDDP cells, was observed at 155 μM cisplatin. When G3361/CDDP cells where pretreated with anti-ABCB5 P-glycoprotein Ab and the highest ABCB5 P-glycoprotein-expressing tumor cell subset depleted, cisplatin resistance reversal was further enhanced (Fig. 6C) and logarithmic cell killing was maintained throughout the tested cisplatin concentration range (LC50 30 μM, LC90 70 μM, LC99 160 μM), with a >1 log enhancement of cell killing (97% of control survivors killed, $p<0.05$) at 200 μM cisplatin.

6. The experimental results set forth in paragraph 5 above demonstrate that ABCB5 is involved in transporting drugs out of cells. As such, an assay designed to identify agents that block the activation of this protein should be useful as a tool for the identification of new agents of potential use during the chemotherapy of cancer patients.

7. I further declare that all statements made herein on the basis of personal knowledge are true, and all statements made on information and belief are believed to be true; and further that any wilful false statements or the like so made are punishable by fine or imprisonment or both under Section 1011 of Title XVIII of the United States Code; and that such wilful false statements may jeopardize the validity of the above-captioned application or any patent issuing thereon.

Respectfully submitted,

09/26/2003
Date

Markus Frank, MD

Appendix I: Figure Legends

Figure 1: Comparison of the human ABCB5 P-glycoprotein primary amino acid sequence with the homologous sequence portions of ABCB1 (MDR1) and ABCB4 (MDR3) P-glycoprotein. Amino acid identity (red), substitution of highly similar (green), similar (purple) or unrelated (black) amino acid residues are illustrated.

Figure 2: ABCB5 P-glycoprotein gene expression. RT-PCR amplification of ABCB5 P-glycoprotein cDNA in G3361 (lane 2) and G3361/CDDP (lane 3) melanoma cells and human epidermal melanocytes (HEM)(lane 6); MCF-7 (lane 4), SCC-25 (lane 5), PBMC (lane 7), molecular size markers (lane 1).

Figure 3: ABCB5 P-glycoprotein expression and topology. **A** Western analysis of ABCB5 P-glycoprotein expression in HEM, G3361 melanoma cells, MCF-7 breast carcinoma control cells and ABCB5 gene-transfected MCF-7 cells, with molecular size markers (kD) shown on the left. **B** Transfection efficiency of lacZ gene-transfected MCF-7 cultures vs. controls, with galactosidase-positive cells identified by blue staining. **C** Flow cytometry analysis of surface anti-ABCB5 mAb staining (solid line) versus isotype control Ab staining (shaded) on control LacZ gene- or ABCB5 gene-transfected MCF-7 cells. **D** Transmembrane helix formation probability of ABCB5 P-gp, as determined using the TMHMM1.O software algorithm for the prediction of transmembrane helix formation in mammalian proteins.

Figure 4: Native ABCB5 P-glycoprotein expression. **A** Flow cytometry analysis of anti-ABCB5 P-glycoprotein mAb staining (solid line) or isotype control Ab staining

(shaded) on HEM or G3361 and G3361/CDDP human melanoma cells. **B** ABCB5 P-glycoprotein expression on unseparated or purified CD133$^+$ HEM (solid line: anti-ABCB5 mAb, shaded: isotype control Ab). **C** ABCB5 P-glycoprotein expression on CD133$^+$ HEM. Indirect immunofluorescence labeling (green, primary anti-ABCB5 mAb followed by FITC-labeled goat anti-mouse secondary Ab) and counterstaining of nuclei with DAPI (blue) revealed cells with four nuclei (panel 1), three nuclei (panel 2), two nuclei (panel 3,4) or single nuclei (panel 5). The left column shows DAPI staining, the middle column shows anti-ABCB5 mAb staining, and the right column shows the merged image (200x magnification). The size bar represents 50 μm.

Figure 5: Flow cytometry analysis of ABCB5 P-glycoprotein-mediated rhodamine-123 and doxorubicin transport function. **A** Rhodamine-123 fluorescence of ABCB5 gene-transfected MCF-7 cells (solid line) versus LacZ gene-transfected MCF-7 control cells (shaded) is shown as a time course (minutes after loading). **B** Doxorubicin fluorescence of anti-ABCB5 mAb-treated G3361 human melanoma cells (solid line) versus control Ab-treated G3361 cells (shaded) immediately upon completion of doxorubicin loading and 30 min mAb incubation.

Figure 6: Effects of ABCB5 P-glycoprotein blockade on cisplatin (CDDP) resistance reversal in G3361/CDDP melanoma cells. **A** CDDP resistance profiles of G3361 (empty circle symbol) and G3361/CDDP (filled circle) cell lines in absence of ABCB5 P-glycoprotein inhibition (surviving cell fraction (mean±SD) versus CDDP concentration (μM)). The LD50 for both cell lines is illustrated (dotted line). **B** CDDP resistance profiles of control (full circle) or anti-ABCB5 P-glycoprotein Ab-treated G3361/CDDP melanoma cells (surviving cell fraction

(mean±SD) versus CDDP concentration (μM)). The LD50 is illustrated by the dotted line. **C** CDDP resistance profiles of unseparated (full circle) or ABCB5 P-glycoprotein$^+$-depleted G3361/CDDP melanoma cells (surviving cell fraction (mean±SD) versus CDDP concentration (μM)). The LD50 is illustrated by the dotted line.

Appendix II: Methodology

Cell culture and isolation: Human epidermal melanocytes (HEM) isolated from the foreskins of healthy donors were purchased from Gentaur, Brussels, Belgium, and cultured in HAM-F10 medium (Clonetics, Walkersville, MD) supplemented with 10% foetal bovine serum (FBS), 6 mM HEPES and 25 μg/ml bovine pituitary extract (BPE) (Gibco), 10 μg/ml insulin (Sigma), 2.8 μg/ml hydrocortisone, 2 mmol/l L-glutamine, 100 IU/ml Penicillin/Streptomycin (Sigma), 0.6 ng/ml basic fibroblast growth factor (bFGF) (Sigma) and 10 nmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma) as previously described(36). The G3361, G3361/CDDP, MCF-7 and SCC25 tumor cell lines were provided by Dr. Emil Frei III, Dana Farber Cancer Institute, Boston, MA and were cultured as described(31). Peripheral blood mononuclear cells (PBMC) were donated by healthy volunteers, and isolated as described(15). CD133$^+$ cell populations were isolated from HEM cultures harvested by EDTA-4Na 0.2 g/l PBS treatment (Versene 1:5000, Invitrogen, Carlsbad, CA), by positive selection using anti-CD133 Ab-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA) and purification in MiniMACS separation columns (Miltenyi Biotec) according to the manufacturer's recommendations. ABCB5 P-glycoprotein$^+$ cells were isolated from HEM cultures by incubation with anti-ABCB5 mAb (20 μg/ml) for 30 min at 4°C, washing for excess antibody removal, incubation with goat anti-mouse Ig-coated magnetic microbeads and subsequent purification in MiniMACS separation columns (Miltenyi Biotec). For depletion of ABCB5$^+$ melanoma cells from G3361/CDDP cultures, melanoma cells were first incubated with anti-ABCB5 P-glycoprotein Ab (1:20 dilution) for 30 min at 4°C, followed by cell washing for excess antibody removal, incubation with goat anti-

mouse Ig-coated magnetic microbeads and subsequent depletion of ABCB5 $^{+}$ cells in MiniMACS separation columns (Miltenyi Biotec).

RNA extraction and RT-PCR: Cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA) followed by isopropanol precipitation and ethanol washing. Each sample was treated with 10 units of Rnase-free Dnase I (Boehringer Mannheim, Germany) and incubated at 37 °C for 30 min followed by phenol/chloroform extraction and ethanol precipitation. Standard cDNA synthesis reactions were performed using 5 μg RNA and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as per manufacturer's instructions. For PCR analysis, 5 μl of diluted first strand product (100 ng of cDNA) was added to 45 μl of a 1x PCR mix (50 pmole of each primer, 1X PCR PreMix C (MasterAmp PCR Optimization Kits, Epicentre, Madison, Wisconsin), 2.5U MasterAmp TAQurate DNA Polymerase Mix (Epicentre) and denatured at 95 °C for 3 min, then cycled 35 times at 94 °C for 1 min, 58 °C for 30 s and 72°C for 3 min, and subsequently extended at 72 °C for 10 min. Reaction products were resolved on a 1% LE agarose gel (Ambion, Austin, TX) and photographed. β-actin primers were used as controls to ensure RNA integrity. The gene-specific oligonucleotide primer pair:

5'-CCTAATTCCTCTAATATCTCTCTGTGAGCC-3' (forward primer) and

5'-TCACTGCACTGACTGTGCATTCAC-3' (reverse primer)

were used for PCR amplification of reverse transcribed total mRNA. The 2906 base cDNA PCR product was sequenced using the amplification primer pair and additional gene-specific sequencing primers:

5'-AGTGGGAAGAGTACGGTAGTCCAGCTTCTG-3';

5'-CCTTTAGGGTCACAATCAAATCTGCACTTCG-3';

5'-GAGCAAAGGTCGGACTACAATCGTG-3';

5'-CCCCTGCTCTGCCGTAAAATAATCC-3';

5'-ATTTTGGGTGTTATTTGCTTTGTCAG-3';

5'-CTGCGGCTGTCTATATTTGGTTTC-3';

5'-CCAGAGGGCATGTTCATAGTTTTTAC-3'; and

5'-ACGGCTGTTGTCACCATAGGC-3';

and the dideoxy chain termination method analyzed by an ABI PRISM 377 DNA Sequencer (Perkin Elmer Life Sciences, Boston, MA).

ABCB5 gene cloning and transfection: For ABCB5 gene transfection experiments, a 2506-base cDNA containing the full-length ABCB5 open reading frame (ORF) was amplified by PCR from the RT product of total HEM RNA using a four base (CACC) 5'-modified forward primer: 5'– CACC AGT GGG AAG AGT ACG GTA GTC CAG CTT CTG-3' and the reverse primer 5'-TCA CTG CAC TGA CTG TGC ATT CAC-3' and the Advantage PCR polymerase mix (Clontech, Palo Alto, CA). The resultant blunt-end PCR product was directionally cloned into the pcDNA3.1D/V5-His-TOPO plasmid vector (Invitrogen). Recombinant plasmids were transformed into and amplified in competent TOP10 *E. coli* cells (Invitrogen) and subsequently purified and sequenced. Recombinant ABCB5 ORF-containing plasmids or pcDNA3.1D/V5-His/lacZ expression control plasmids containing the *E. coli* lacZ gene encoding β-galactosidase (Invitrogen) were transfected into cultured ABCB5 non-expressing MCF7 breast cancer cells using relative quantities of plasmid and Lipofectamine 2000 reagent (Invitrogen) as recommended by the manufacturer. ABCB5 gene expression was assessed at 48h by Western analysis and flow cytometry. As a control, pcDNA3.1D/V5-His/lacZ control plasmid-transfected MCF-7 cancer cells were assayed at 48h for β-galactosidase expression using the β-Gal Staining Kit (Invitrogen) and brightfield microscopic examination.

Antibodies: For anti-ABCB5 monoclonal antibody (mAb) production, a 16-mer peptide (R-F-G-A-Y-L-I-Q-A-G-R-M-T-P-E-G) derived from the extracellular loop-associated amino acid residues 493-508 was synthesized and conjugated to the carrier protein BSA via a cystein

residue added for coupling purposes. The immunogen (100 μg) was administered subcutaneously to Balb/c mice during primary immunization, and mice received booster immunizations using 100 μg of immunogen at 4 and 8 weeks. Mouse sera were screened for specific-antibody production against the immunogen by ELISA, and mice were sacrificed and splenocytes collected for fusion with F/O myeloma cells when test bleed serum titers were >1:80000. Specific antibody-secreting fusion products were subcloned to monoclonality by limiting dilution, screened for reactivity with native ABCB5 P-glycoprotein by Western analysis and flow cytometry of ABCB5 P-glycoprotein-expressing HEM and ABCB5 P-glycoprotein gene-transfected, at baseline non-expressing MCF-7 breast cancer cells. The Ig isotypes of mAbs secreted by selected clones was determined and spinner flask-produced mAbs were purified by Protein A affinity chromatography. The IgG1 anti-ABCB5 P-gp mAb UG3C2-2D12 was used in the here-reported studies, and the MOPC-31C mouse isotype control mAb (PharMingen, San Diego, CA) was used as a control. In those experiments were polyclonal anti-ABCB5 P-glycoprotein antibodies were used, mouse antisera were collected for experimental use when test bleed serum titers were >1:80000 as determined by ELISA, and pre-immunization mouse sera were collected for use as controls.

Western analysis: For western analysis, whole cell lysates were mixed with 2X sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 4% sodium dodecyl sulfate SDS), and 0.0025% bromophenol blue), heated at 100 °C for 5 minutes, and run on 4-15% continuous gradient polyacrylamide gels (Bio-Rad, Hercules, CA.) with Tris-glycine-SDS running buffer (Bio-Rad). Separated proteins were transferred to a PVDF membrane (NEN Life Science Product, Boston, MA). Membranes were washed and blocked with PBS-Tween 20 containing 5% dry milk and then coated with either anti-ABCB5 mAb or mouse isotype control antibody. After washings, the membranes were incubated with peroxidase-linked secondary goat anti-

mouse antibody and the reactive bands were detected by the addition of chemoluminescent substrate (Pierce, Rockford, IL).

Flow cytometry: HEM and purified CD133$^+$ HEM, G3361 and G3361/CDDP human melanoma cells, and untreated, ABCB5 gene-transfected or control lacZ gene-transfected MCF-7 breast cancer cells were analyzed for surface ABCB5 P-glycoprotein expression by incubation of 5 x 10^5 cells for 60 min at 4 °C with anti-ABCB5 mAb or isotype control mAb (10 μg/ml), followed by counterstaining with FITC-conjugated goat anti-mouse Ig Ab (PharMingen, San Diego, CA) as described(15). Analysis of cell surface marker expression was performed by single color flow cytometry as described(15). PE-conjugated anti-CD133 mAb (clone AC133/2) and anti-CD34 mAb were obtained from Miltenyi.

Fluorescence microscopy: To visualize ABCB5 P-glycoprotein expression on HEM, CD133$^+$ HEM were purified as above and cultured for 24h in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) at 37 °C in a humidified 5 % CO_2 atmosphere. Cells were then fixed in methanol for 3 min, washed twice in PBS and incubated with anti-ABCB5 mAb or isotype control Ab (10 μg/ml) at 4 °C for 30 min. After washing, cells were incubated with FITC-conjugated goat anti-mouse secondary antibody for 30 min at 4 °C, washed, followed by counterstaining of nuclei with DAPI (Molecular Probes, Eugene, OR). Fluorescence staining was analyzed by fluorescent microscopy using a Mercury-100 Watts fluorescent light source (Microvideo Instruments, Avon, MA) attached to a Nikon Eclipse TE 300 microscope (Nikon Instruments, Melville, NY) with the use of separate filters for each fluorochrome. Images were obtained using a Spot digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) and the Spot 3.3.2. software package were imported into Adobe Photoshop (Adobe Systems, Mountain View, CA) and processed.

Transport studies: For rhodamine-123 transport studies, ABCB5 gene- or control LacZ gene-transfected MCF-7 cells (2 x 10^6) were incubated with rhodamine-123 (1 μg/ml) (Molecular Probes, Eugene, OR) for 60 min at 37 °C and 5% CO_2. Subsequently the cells were washed and serial fluorescence measurements acquired by flow cytometry at the FL1 emission spectrum on a Becton Dickinson FACScan. For doxorubicin transport studies, human G3361 melanoma cells (8 x 10^5) were incubated with doxorubicin (1 μg/ml) (Sigma) for 30 min at 37 °C and 5% CO_2. Cells were subsequently washed and then incubated with anti-ABCB5 mAb or control Ab (50 μg/ml) for 30 min, followed by fluorescence measurements acquired by flow cytometry at the FL2 emission spectrum on a Becton Dickinson FACScan.

Cell viability assays: To determine the cisplatin resistance profile of G3361 and G3361/CDDP human melanoma cells and to examine the effects of ABCB5 P-glycoprotein blockade on cisplatin resistance reversal, freshly harvested subconfluent cell cultures were seeded at 10^4 cells/well in flat-bottom 96-well culture plates in 100 μl medium. Following a 24h incubation in the presence or absence of 1:20 diluted anti-ABCB5 P-glycoprotein Ab or control serum, cisplatin was added at 0, 20, 50, 100 or 200 μM final concentrations for 24h. Alternatively, G3361/CDDP cultures depleted of the ABCB5 P-glycoprotein high-expressing subset were seeded, incubated for 24h to facilitate cell attachment and subsequently treated with cisplatin for 24h in an identical manner. Following cisplatin treatment, the MTT assay for cell viability was performed. Briefly, 10 μl of MTT reagent (R&D systems, Minneapolis, MN) were added per well for 2h at 37 °C followed by addition of 100 μl dye-solubilizing detergent reagent. When formazan dye crystals were completely solubilized after 12 h incubation, absorbance was read at a wavelength of 595 nm in a spectrophotometer and the surviving cell fraction determined from the absorbance ratios of cisplatin-treated versus untreated cultures with blanks subtracted.

Appendix III: References

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Figure 1

```
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NIDGQDIRNFNVNYLREIIGVVSQEPVLFSTTIAENICYGRGNVTMDEIK

RAAREANAYDFIMEFPNKFNTLVGEKGAQMSGGQKQRIAIARALVRNPKI
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KAVKEANAYEFIMKLPQKFDTLVGERGAQLSGGQKQRIAIARALVRNPKI

LILDEATSALDSESKSAVQAALEKASKGRTTIVVAHRLSTIRSADLIVTL
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LLLDEATSALDTESEAEVQAALDKAREGRTTIVIAHRLSTVRNADVIAGF

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TYSTERKTNSLPL--HSVKSIKSDFIDKAEESTQ--SKEISLPEVSLLKI
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FILRGLSLSIERGKTVAFVGSSGCGKSTSVQLLQRLYDPVQGQ-------
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PQILLLDEATSALDTESEKVVQEALDKAREGRTCIVIAHRLSTIQNADLI

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Figure 2

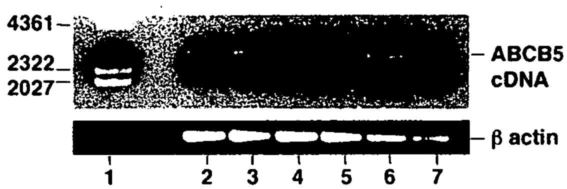


Figure 3

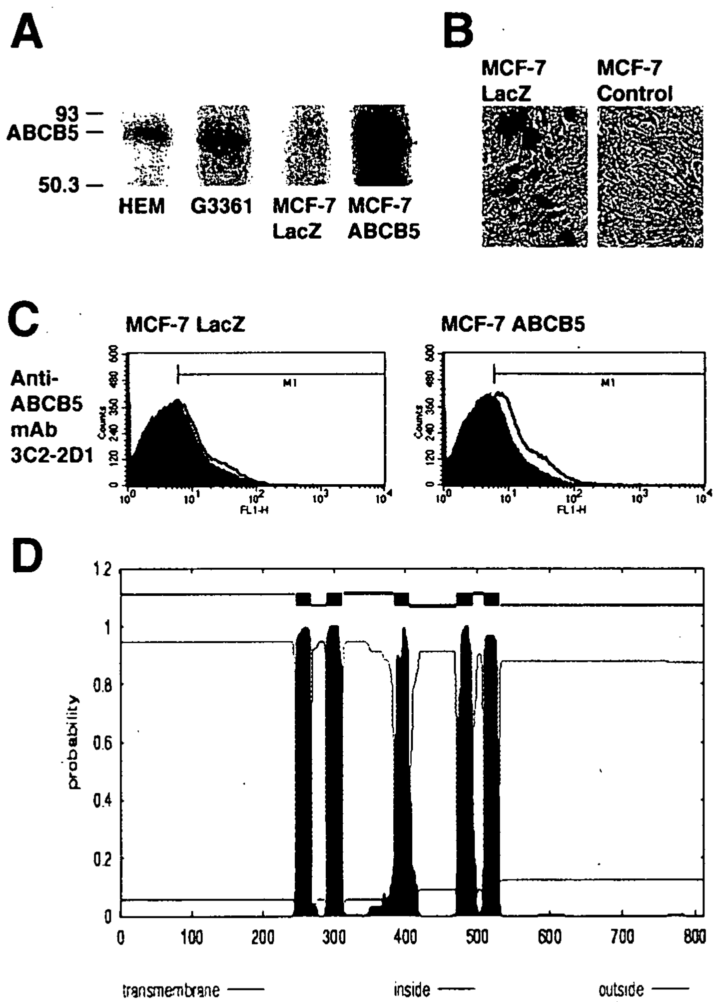

A
93
ABCB5
50.3
HEM
G3361
MCF-7 LacZ
MCF-7 ABCB5
B
MCF-7 LacZ
MCF-7 Control
C
MCF-7 LacZ
MCF-7 ABCB5
Anti-ABCB5 mAb 3C2-2D1
M1
Counts
FL1-H
D
probability
transmembrane
inside
outside

Figure 4

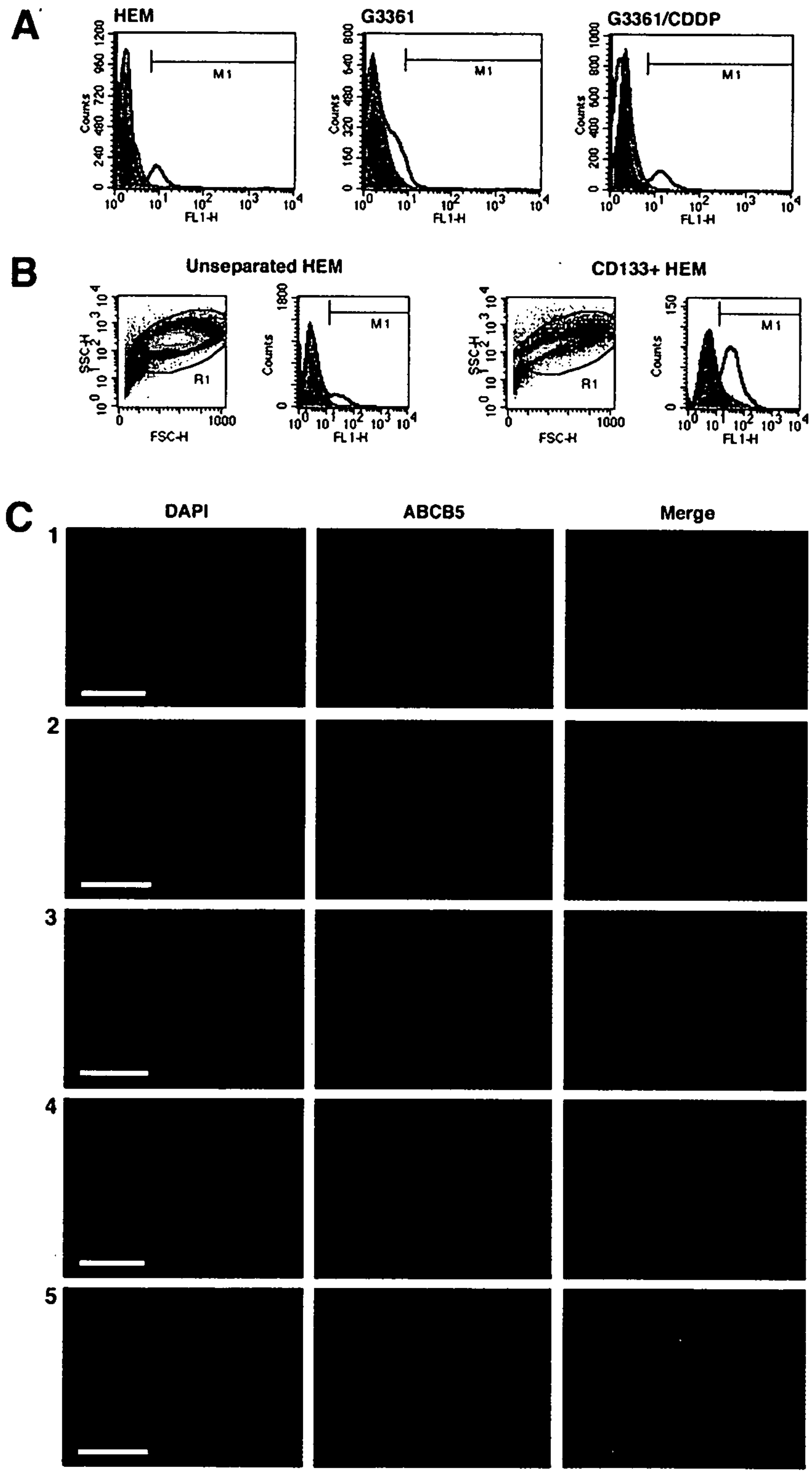
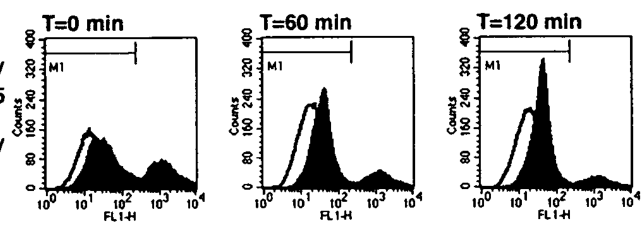

A
HEM
G3361
G3361/CDDP
M1
Counts
FL1-H
B
Unseparated HEM
CD133+ HEM
SSC-H
FSC-H
R1
Counts
FL1-H
C
DAPI
ABCB5
Merge
1
2
3
4
5

Figure 5

A

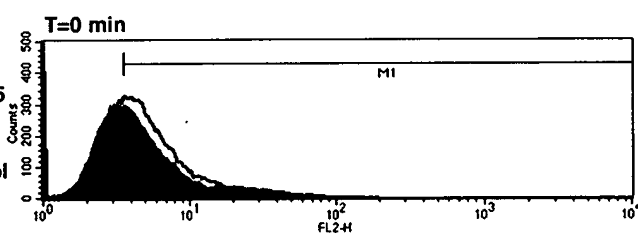
MCF-7/
ABCB5
Vs.
MCF-7/
LacZ



Rhodamine-123 Fluorescence

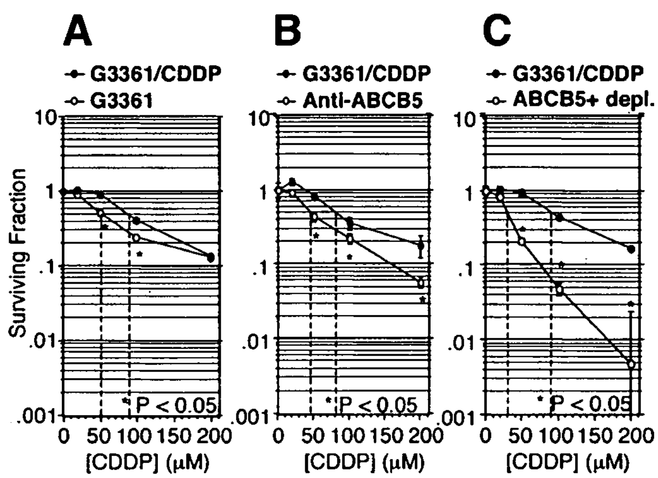
B

G3361
Anti-
ABCB5
mAb
vs.
Control



Doxorubicin Fluorescence

Figure 6


A
G3361/CDDP
G3361
B
G3361/CDDP
Anti-ABCB5
C
G3361/CDDP
ABCB5+ depl.
Surviving Fraction
10
1
.1
.01
.001
0 50 100 150 200
[CDDP] (μM)
* P < 0.05

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Place of Birth: Kirchheim unter Teck, Germany

Education:

1989 A.B.	Harvard University
1992 M.D.	University of Heidelberg School of Medicine, Germany

Postdoctoral Training:

Internships and Residencies:

1993-1994	Intern in Medicine, Ludwig-Maximilians-Universität, München, Germany
1994-1997	Resident, Internal Medicine, Albert Einstein College of Medicine, Bronx, N.Y.

Clinical and Research Fellowships:

1992-1993	Doctoral Research Fellow, German Cancer Research Center, Heidelberg, Germany
1997-2001	Clinical Fellow in Medicine, Renal Division, Brigham and Women's Hospital, Boston, MA.
1998-2001	Research Fellow in Medicine, Renal Division, Brigham and Women's Hospital, Boston, MA.
1998-2001	Research Fellow in Medicine, Division of Nephrology, The Children's Hospital, Boston, MA.

Licensure and Certification:

1997	Massachusetts Board of Registration in Medicine License Registration
1997	Diplomate, American Board of Internal Medicine

Academic Appointments:

1987-1988	Instructor in Anatomy, University of Heidelberg School of Medicine, Germany
1997-2001	Clinical Fellow in Medicine, Harvard Medical School, Boston, MA.
1998-2001	Research Fellow in Medicine, Harvard Medical School, Boston, MA.
2001-present	Instructor in Medicine, Harvard Medical School, Boston, MA.

Hospital Appointments:

1993-1994	Intern in Medicine, Medizinische Poliklinik, Ludwig-Maximilians Universität, München, Germany
1994-1995	Intern in Medicine, Albert Einstein College of Medicine, Bronx, N.Y.
1995-1997	Resident, Internal Medicine, Albert Einstein College of Medicine, Bronx, N.Y.
1997-2001.	Clinical Fellow in Medicine, Division of Nephrology, Brigham and Women's Hospital, Boston, MA.
1998-present	Staff Physician, Department of Medicine, Veterans Administration Medical Center, Boston, MA.
2001-present	ResearchAssociate, Division of Nephrology, Children's Hospital, Boston, MA.
2001-present	Associate Physician, Renal Division, Brigham and Women's Hospital, Boston, MA.

Awards and Honors:

1984-1992	Scholar, German National Scholarship Foundation (Top 1% of University Class)
1988/89	Harvard College Scholarship (Academic Achievement of High Distinction)
1989	Bachelor of Arts degree awarded 'magna cum laude', Harvard University
1991-1992	Scholarship, German Academic Exchange Service
1992	Doctor of Medicine degree awarded 'magna cum laude', University of Heidelberg
1997	Diplomate, American Board of Internal Medicine
1999	American Society of Transplantation – Novartis Fellowship in Transplantation Award
2000	Young Investigator Award, American Society of Transplantation
2000	Travel Award, International Pediatric Transplant Association
2001	AST Council's Faculty Grant Award, American Society of Transplantation
2001	Theodore Steinman Award in Clinical Research, National Kidney Foundation
2002	Mentored Clinical Scientist Award, National Institutes of Health
2003	Developmental Project Grant Award, Special Program of Research Excellence (SPORE) in Skin Cancer, Division of Dermatology, Brigham and Women's Hospital, Boston, MA.

Memberships, Offices and Committee Assignments in Professional Societies:

1995-present	Member, American Medical Association
1995-present	Member, American College of Physicians
1998-present	Member, Massachusetts Medical Society (Member, Committee on Young Physicians 2001-2002)
1999-present	Member, American Society of Nephrology (Abstract Review Committee ASN Annual Meeting 2003)
2000-present	Member, American Society of Transplantation

	(Abstract Review Committee American Transplant Congress 2002 and 2003; Invited Scientific Session Chair: Lymphocyte Activation and Down Regulation, ATC 2002; Costimulation, ATC 2003)
2000-present	Member, The Transplantation Society
2000-present	Member, NKF
2000-present	Ad hoc referee: Blood, Journal of the American Society of Nephrology, Kidney International, Journal of Immunology
2001-present	Member, International Society of Comparative Oncology
2003-present	Member, American Society of Human Genetics
2003-present	Member, American Society of Biochemistry and Molecular Biology

Major Research Interests:

1. Immunoregulatory Role of P-glycoprotein
2. Role of ABC transporters in T cell function and APC differentiation in autoimmunity and allograft rejection
3. P-glycoprotein-mediated cancer multidrug resistance
4. Role of ABC Transporters in Adult Stem Cell Function

Self Report of Teaching:

10/26/01	Renal Grand Rounds, Brigham and Women's Hospital, Boston, MA. (Title: Targeting Adhesion Molecules in Allotransplantation)
2002-2003	Thesis advisor, Honors Dissertation Sam Behjati, St. Hugh's College, Oxford University, UK

Local Contributions:

University of Heidelberg School of Medicine

1987-1988 Gross Anatomy

Section Leader and Instuctor in Gross Anatomy Laboratory
10 medical students (section); 10 hours / week during two consecutive semesters

Bibliography:

Original Reports:

1. Frank NY, Pendse S, Lapchak P, Shlain D, Doeing C, Sayegh MH, **Frank MH**. Regulation of progenitor cell fusion by ABCB5 P-glycoprotein, a novel human ATP-binding cassette transporter. Journal of Biological Chemistry, Sep 7 2003; 10.1074/jbc.M308700200
2. **Frank MH**, Denton MD, Alexander SI, Khoury SJ, Sayegh MH, Briscoe DM. Specific MDR1 P-glycoprotein blockade inhibits human alloimmune T cell activation in vitro. Journal of Immunology 2001; 166: 2451-2459
3. **Frank MH**, Pomer S. IFN-alpha differentially affects proliferation of two human renal cell carcinoma cell lines differing in the P-glycoprotein-associated multidrug resistance phenotype. Journal of Cancer Research and Clinical Oncology 1999; 125(2): 117-120.
4. Kellner H., Liegl U., **Frank M.**, Zoller W.G. Reversible esophageal dysfunction as a side effect of levodopa. Bildgebung 1996; 63 (1): 48-50.

Reviews:

1. Pendse S, Sayegh MH, **Frank MH**. P-glycoprotein – a novel therapeutic target for immunomodulation in clinical transplantation and autoimmunity? Current Drug Targets 2003; 4:469-476 (Invited Review).
2. Pendse S, Briscoe DM, **Frank MH**. P-glycoprotein and alloimmune T cell activation. Clinical and Applied Immunology Reviews 2003; 4 (1):3-14 (Invited Review)

Proceedings of Meetings:

1. Frank NY, Pendse S, Lapchak PH, Doeing C, Sayegh MH, **Frank MH**. Regulation of progenitor cell fusion by ABCB5 P-glycoprotein, a novel human ATP-binding cassette transporter. American Society of Human Genetics 53rd Annual Meeting 2003 (accepted).

2. Izawa A, Frank NY, Pendse S, Sayegh MH, **Frank MH**. P-glycoprotein blockade prolongs murine cardiac allograft survival. American Heart Association Scientifc Sessions 2003 (accepted).
3. Izawa A, Pendse S, Sayegh MH, **Frank MH**. MDR1 Specific pharmacologic P-glycoprotein blockade prolongs murine cardiac allograft survival. American Journal of Transplantation 2003; 3 (Supplement 5): 447: Abstract# 1153.
4. Pendse S, Behjati S, Sayegh MH, **Frank MH**. MDR1 P-glycoprotein is a dendritic cell/macrophage differentiation switch in antigen presenting cell maturation. American Journal of Transplantation 2003; 3 (Supplement 5): 340: Abstract# 732.
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6. Pendse S, Sayegh, MH, **Frank MH**. A critical role for P-glycoprotein in early cytokine-dependent dendritic cell maturation. American Journal of Transplantation 2002; 2 (Supplement 3): 265: Abstract# 504.
7. **Frank MH**, Sayegh MH, Briscoe DM. P-glycoprotein blockade inhibits alloimmune T cell responses via both CD4+ T cell- and CD14+ APC-dependent activation pathways. American Journal of Transplantation 2001; 1 (Supplement 1): 267: Abstract# 527.
8. **Frank MH**, Sayegh MH, Briscoe DM. P-glycoprotein function in alloimmune lymphocyte/endothelial cell interactions. Journal of the American Society of Nephrology 2000; 11:661A: A3485.
9. **Frank MH**, Denton MD, Alexander SI, Khoury SJ, Sayegh MH, Briscoe DM. P-glycoprotein blockade inhibits alloimmune T cell activation in vitro. XVIII International Congress of the Transplantation Society, Rome, Italy, 2000; p161: Abstract POO26W.
10. **Frank MH**, Sayegh MH, Briscoe DM. P-glycoprotein function in human alloimmune interactions. Pediatric Transplantation 2000; 4 (Supplement No. 2): 108: Abstract 0178
11. **Frank MH**, Sayegh MH, Briscoe DM. P-glycoprotein function in human alloimmune interactions. Transplantation 2000; 69(8): S131: A78
12. **Frank MH**, Chitnis T, Abdallah KA, Sayegh MH, Khoury SJ. Tamoxifen inhibits disease expression in murine experimental autoimmune encephalomyelitis. Neurology 2000; 54 (7) Suppl 3: A61-A61

13.**Frank MH**, Denton MD, Alexander SI, Khoury SJ, Sayegh MH, Briscoe DM. P-glycoprotein is functional in antigen- and alloantigen-dependent T cell activation. Journal of the American Society of Nephrology 1999; 10:701A: A3551.

14.Denton MD, **Frank MH**, Waaga AM, Geehan C, Sayegh MH, Briscoe DM. Human endothelial cells facilitate indirect allorecognition. Journal of the American Society of Nephrology 1999; 10:699A: A3540.

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17.Lu R, **Frank M**, Schuster VL. Genomic Cloning of the Human Prostaglandin (PG) Transporter "PGT" Gene. Journal of the American Society of Nephrology 1996; 7: A1997.

18.**Frank MH**, Shedd SF, Lutz NW, Pomer S, Hull WE. ^{31}P Nuclear Magnetic Resonance Spectroscopy Study of Multidrug Resistance in Human Renal Cell Carcinoma. In: Society of Magnetic Resonance in Medicine 12th Annual Conference. New York: Society of Magnetic Resonance in Medicine, 1993: 1015.

19.Shedd SF, Lutz NW, **Frank MH**, Hull WE. Choline- and Ethanolamine- Dependent Regulation of Phosphomonoester and Uridine diphospho-hexose Metabolism in a Human Colon Cancer Cell Line. In: Society of Magnetic Resonance in Medicine 12th Annual Conference. New York: Society of Magnetic Resonance in Medicine, 1993: 262.

Books and Monographs:

1. **Frank MH** and Sayegh MH (2001). Immunology and Tolerance: In: Transplantation 2001 (New York, N.Y.: Lippincott Williams & Wilkins Healthcare).
2. **Frank MH** and Sayegh MH (2001). Tolerance: is it time to move to the clinic? In: Current and Future Immunosuppressive Therapies Following Transplantation.

Mohamed Sayegh and Giuseppe Remuzzi, Editors, (Dordrecht, The Netherlands, Kluwer Academic Publishers), pp. 293-313.

3. **Frank MH** and Briscoe DM (2001). Monoclonal Antibody Targeting of Adhesion Molecules. In: Current and Future Immunosuppressive Therapies Following Transplantation. Mohamed Sayegh and Giuseppe Remuzzi, Editors, (Dordrecht, The Netherlands, Kluwer Academic Publishers), pp. 249-263.
4. **Frank MH** and Sayegh MH (2000). Immunology and Tolerance. In: Transplantation 2000: Transplantation at the Millennium (New York, N.Y.: Lippincott Williams & Wilkins Healthcare).
5. **Frank MH** and Sayegh MH (1999). Transplantation Tolerance. In: Transplantation 1999: Progress on the Cusp of the Millennium (New York, N.Y.: Lippincott Williams & Wilkins Healthcare).
6. **Frank MH.** ^{31}P-NMR-spektroskopische Studien zur Multidrug-Resistenz des menschlichen Nierenzellkarzinoms [Dissertation]. Heidelberg, Germany: Ruprecht-Karls-Universität Heidelberg, 1995. 79pp.

Patents:

1. **Frank MH,** Sayegh MH. A gene encoding a multidrug resistance human P-glycoprotein homologue on chromosome 7p15-21 and uses thereof (Appl. No.: 09/873,409).
2. **Frank MH.** Tumor suppressors associated with human chromosome 21q22 (Appl. No.: 10/303,995).